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Antitumor Effects of Human Recombinant Interferon-γ and Tumor Necrosis Factor on Five Cervical Adenocarcinoma Cell Lines, *in Vivo* and *in Vitro*

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We examined the antitumor effects of recombinant interferon-γ (IFN-γ) and tumor necrosis factor (TNF) on cervical adenocarcinoma cell lines, *in vitro* and *in vivo*. Four of five cell lines showed a high sensitivity to IFN-γ, *in vitro*. One of five cell lines showed a remarkable sensitivity to TNF, *in vitro*. Only one cell line resistant to both IFN and TNF was derived from a well-differentiated adenocarcinoma of endocervical type. Experiments using nude mice bearing transplanted tumors revealed that these cytokines were also effective against tumors *in vivo*. All these observations suggest that IFN-γ or TNF can have positive effects in the treatment of patients with adenocarcinoma of the uterine cervix.

INTRODUCTION

Adenocarcinoma of the uterine cervix usually has a less favorable prognosis than does squamous carcinoma [1,2]. The different prognosis of these two histologic types has been attributed to the finding that adenocarcinoma tends to metastasize earlier to lymphnodes and is less sensitive to radiotherapy and to chemotherapy. In the search for effective antitumor agents for treating patients with adenocarcinoma of the uterine cervix, cytokines, such as interferons (IFNs) and tumor necrosis factor (TNF), are worthy of consideration.

Interferons inhibit the multiplication of both diploid and transformed human cells *in vitro*, and this growth inhibition is the direct effect of the IFNs [3–9]. This property provides the basis for the clinical use of IFNs to treat human malignancies. IFNs have been classified into three different types, α, β, and γ, all which have different characteristics of anticellular activity [9–13]. IFN-γ, more than IFN-α or IFN-β, was seen to be effective in inhibiting growth of tumor cells [9,11,14,15].

Tumor necrosis factor was first recognized to induce hemorrhagic necrosis of tumors *in vivo* and to have cytotoxic effects on mouse and human tumor cells *in vitro* by Carswell *et al.* [16]. As this agent has no apparent cytotoxic effect on normal cells, it is worthy of extensive investigation.

The present study was done to determine whether IFN-γ and TNF would have adequate antitumor effects on cervical adenocarcinoma cell lines. The clinical applicability was also given attention.

METHODS

Cells. Five human cervical adenocarcinoma cell lines designated as HeLa, JSK-1, TMCC-1, CAC-1, and OMC-4 were tested for their sensitivities to IFN-γ and TNF. Cell lines OMC-4, CAC-1, and TMCC-1 were established by Dr. T Yamada (Osaka Medical College, Osaka, Japan), Dr. O Hayakawa (Sapporo Medical College, Sapporo, Japan), and Dr. M Sakamoto (Tokyo Medical College, Tokyo, Japan), respectively. These lines were derived from well, moderately, and poorly differentiated adenocarcinoma of endocervical type, respectively. Cell line JSK-1 was established by Dr. H Sasaki (The Jikei University School of Medicine, Tokyo, Japan) and was derived from a well-differentiated adenocarcinoma of endometrioid type.

Cell cultures. All the cell lines were passaged *in vitro* at intervals of 7–14 days. JSK-1, TMCC-1, CAC-1, and OMC-4, cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 20% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of kanamycin. HeLa cells were cultured in MEM with 10% FCS.

Transplantation into nude mice. Balb/c female nude mice, approximately 6 weeks old and bred in a Specific

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Pathogen-Free Unit, were used for these experiments. The cultured cells were dispersed into single cells using a 0.025% trypsin–0.025% EDTA solution. Cells (1×10^7) were suspended in 0.1 ml of culture medium and injected into the right dorsam of each mouse. Tumor size was measured weekly by two dimensions (long and short diameters). Estimated tumor volume was calculated with the formula

$$\text{Estimated tumor volume } (mm^3) = (\text{Long diameter } (mm)/2) \times (\text{Short diameter } (mm)/2)^2 \times 4/3\pi.$$

Interferon and tumor necrosis factor. Human recombinant IFN-γ was provided by Toray Industries (Tokyo, Japan). TNF was provided by Asahi Kasei Industries (Tokyo, Japan). The IFN-γ used in our experiments contained 1×10^6 IU/ml and the TNF contained 5×10^5 IU/ml, respectively, and each cytokine used was of the same lot number.

Anticellular activities of cytokines. For the study of growth-inhibitory activity of IFN-γ or TNF, the slow-growing cells (4×10^4) or other cells (2×10^4) were seeded in the presence or absence of these cytokines in separate plastic petri dishes (25 mm in diameter; Falcon Plastics, Oxford, CA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air and were harvested by 0.025% trypsin–0.025% EDTA on subsequent days to count the numbers. The number of cells was expressed as the mean cell number calculated from duplicate cultures at each concentration. No medium change was carried out before harvesting the cells. The growth-inhibitory activity of cytokines was expressed as the IC_{50} (cytokine concentration required for 50% growth inhibition), calculated from the mean cell number at each concentration.

Cytokine treatment against tumors in nude mice. Cytokine doses were determined by the maximum doses that can induce no apparent toxicity in nude mice and the schedule and the route of injection were determined according to the previous paper [17].

For IFN-γ treatment, 30 female nude mice were separated into three groups and the injection of IFN was initiated after size of the transplanted tumor exceeded 5 mm in diameter. Group 1: 1×10^5 IU of IFN-γ in 0.1 ml of normal saline was administered daily directly into the tumor (i.t.) for 2 weeks. Group 2: The same amount of IFN was administered daily intramuscularly (i.m.) for 2 weeks. Group 3: Normal saline was injected daily i.t. for 2 weeks, as a control.

For TNF treatment, 50 female nude mice were separated into five groups and TNF administration was also begun after the tumor size exceeded 5 mm in diameter. Group 1: 2×10^4 IU of TNF in 0.1 ml of normal saline was administered directly i.t. three times a week for 2 weeks. Group 2: 2×10^4 IU of TNF was administered i.t. weekly for 2 weeks. Group 3: 2×10^3 IU of TNF was injected directly i.t. three times a week for 2 weeks. Group 4: 2×10^3 IU of TNF was injected directly i.t. weekly for 2 weeks. Group 5: Normal saline was administered i.t. three times a week for 2 weeks, as a control.

Estimated mean tumor volume, obtained from each experimental group, was statistically analyzed using the *t* test.

Evaluation of histologic changes by cytokine treatment. All the animals were killed 32 days after the transplantation, and the tumors were excised and examined histologically.

TABLE 1
Growth Inhibitory Activities of IFN-γ and TNF against Human Cervical Adenocarcinoma Cell Lines

Cell line	IC_{50} (IU/ml)	
	IFN-γ	TNF
HeLa	<10	$>10^3$
JSK-1	<10	$>10^3$
TMCC-1	<10	$>10^3$
CAC-1	70	<10
OMC-4	$>10^3$	$>10^3$

RESULTS

Anticellular Activities of IFN-γ and TNF in Vitro

On the sixth culture day, comparative antiproliferative activities of IFN-γ and TNF on these cell lines were expressed as the IC_{50} in Table 1. Three cell lines (HeLa, TMCC-1, and JSK-1) were highly sensitive to IFN-γ (IC_{50} < 10 IU/ml) and another cell line (CAC-1) was also sensitive to this IFN (IC_{50} < 100 IU/ml), while the other cell line (OMC-4) had no sensitivity to this IFN. Although JSK-1 cells showed a high sensitivity to IFN, on the basis of the value of IC_{50}, the anticellular activity was not in accord with the IFN concentration, as shown in Fig. 1A. On the other hand, only one cell line (CAC-1) showed a remarkable sensitivity to TNF and the other four cell lines were all resistant to this agent (Table 1, Fig. 1B).

Thus four of five cell lines derived from cervical adenocarcinomas were sensitive to either IFN-γ or TNF.

Antitumor Effect of IFN-γ

To test the antitumor effects of IFN-γ *in vivo*, we used the TMCC-1 cell line, which is highly sensitive to IFN-γ *in vitro*. Figure 2 shows the growth curve of the solid tumors after inoculation of 1×10^7 TMCC-1 cells into nude mice and also the effect of IFN. The transplanted tumors in the control group grew to over 5 mm in di-

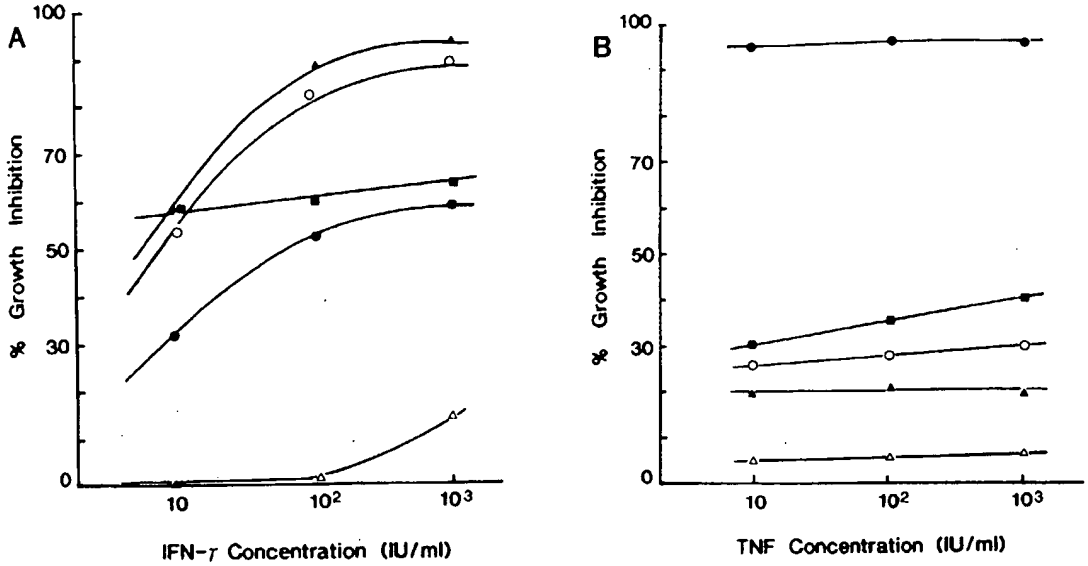

FIG. 1. Anticellular effect of IFN-γ (A) and TNF (B) on the growth of cervical adenocarcinoma cell lines. Cells (2–4 × 10^4) were seeded in plastic dishes and various concentrations of IFN-γ and TNF were added simultaneously on Day 0. Cells were harvested by trypsinization and counted in a Coulter counter on Day 6. (○) HeLa, (■) JSK-1, (▲) TMCC-1, (●) CAC-1, (△) OMC-4.

ameter by Day 14 and to approximately 10 mm by Day 28. Both intramuscular and intratumoral administration of IFN-γ showed remarkable antitumor effects. In mice in Group 2 given a daily i.m. injection of IFN, there was a marked shrinkage of the transplanted tumors ($P < 0.01$) and in those in Group 1, which received a daily i.t. administration, the tumors almost completely regressed by Day 32 after the transplantation ($P < 0.001$). Intratumoral direct injection was more effective than the i.m. route for the inhibition of growth of the transplanted tumors (Fig. 2, Table 2).

Antitumor Effect of TNF

The antitumor activities of TNF on CAC-1 cells transplanted into nude mice were examined in relation to the dose of TNF administration. Figure 3 shows the growth curve of the transplanted tumors after treatment with TNF.

The transplanted tumors in the control group grew to approximately 5 mm in diameter by Day 12 and to more than 10 mm by Day 25. Groups 1 and 2, in which a high dose of TNF (2 × 10^4) was injected directly into tumors three times a week and weekly, respectively, exhibited the most remarkable tumor regression ($P < 0.01$); however, differences in antitumor effect were not observed between the two groups (Table 3). In Group 3, in which a low dose of TNF (1 × 10^3) was administered three times a week, there was also evidence of tumor regression ($P < 0.01$). Tumor growth also seemed to be inhibited by the weekly administration of a low dose of TNF (Group 4), but the difference was not statistically significant.

Histopathologic Survey of the Transplanted Tumors

The cell line TMCC-1 used for IFN treatment was originally derived from a poorly differentiated adenocarcinoma of endocervical type. Analysis of sections of the transplanted tumors from the control group demonstrated a histologic pattern compatible with that seen in the original tumor, while tumors in the IFN-treated groups regressed, leaving only a fibrosis.

Cell line CAC-1 was used to test the effects of TNF. This line was derived from a moderately differentiated adenocarcinoma of endocervical type. Unlike the original tumor, differentiation of the tumors formed in nude mice was poor rather than moderate. Here, TNF treatment led to an extensive tumor necrosis or hemorrhage and there was a remarkable infiltration of neutrophils.

COMMENTS

Effective treatments for patients with an adenocarcinoma of the uterine cervix have eluded clinicians. Even radiotherapy and chemotherapy plus combinations of conventional antitumor agents are not effective for patients with an advanced disease.

We reported that a cell line derived from squamous cell carcinoma of the uterine cervix was remarkably sensitive to IFNs [9,17,18]. We have found no reports on the antitumor effects of IFNs or other antitumor agents

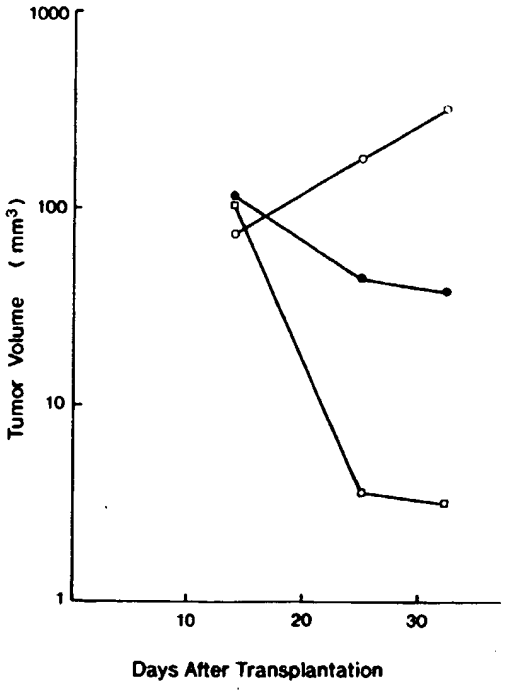

FIG. 2. Antitumor effect of IFN-γ on the growth of TMCC-1 in nude mice. 1×10^5 IU IFN-γ was administered daily directly into tumor (Group 1, □) or intramuscularly (Group 2, ●) for 2 weeks starting 2 weeks after transplantation. Saline was injected intramuscularly daily for 2 weeks into the control (Group 3, ○).

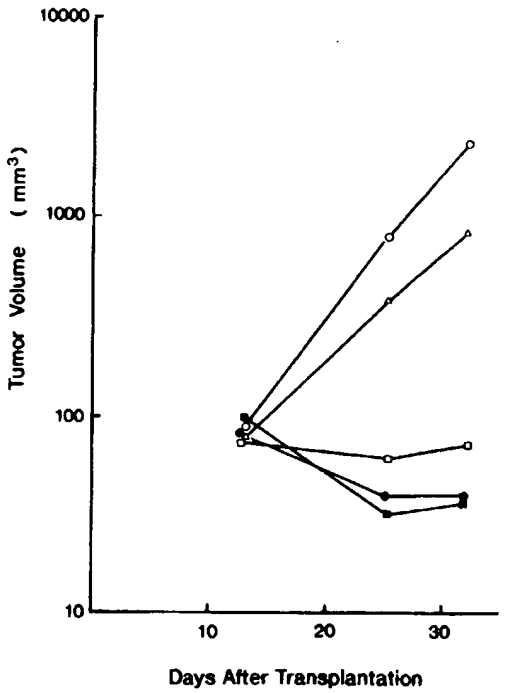

FIG. 3. Antitumor effect of TNF on the growth of CAC-1 cells in nude mice. 2×10^4 IU TNF was administered directly into the tumor (i.t.) three times a week (Group 1, ●) or weekly (Group 2, ■) or 2 weeks, while lower dose (2×10^3 IU) was administered i.t. three times a week (Group 3, □) or weekly (Group 4, △) for 2 weeks. Normal saline was injected i.t. three times a week for 2 weeks as a control (Group 5, ○).

in vitro or *in vivo* against adenocarcinoma of the uterine cervix.

In the present study, we obtained evidence of the positive effects of IFN-γ or TNF *in vitro* as well as *in vivo* against five cell lines derived from adenocarcinoma of the uterine cervix. We also observed the tendency that the poorer the grade of tumor differentiation, the higher the effect of IFN against this carcinoma, although this observation was based on only three cell lines. This is in accord with the clinical observation that extremely well-differentiated adenocarcinoma (so-called adenoma malignum) is resistant to treatment and carries the poorest prognosis. Therefore, the antitumor effect of IFN might derive from the differentiation potential of this agent. We have already noted this potential in a cervical squamous carcinoma cell line, *in vitro* and *in vivo* [17,18], and in the case of a cervical intraepithelial neoplasia [19].

Although the mechanism of antitumor effect of these cytokines is not clear, our results do suggest that these cytokines can be considered for the treatment of adenocarcinoma of the uterine cervix.

TABLE 2
Antitumor Activities of IFN-γ against Transplanted TMCC-1 Tumors

Treatment	No. of animals	Estimated tumor volume (mean ± SD, mm³)		
		Day 14	Day 25	Day 32
Group 1	10	109 ± 50	3.6 ± 2.4*	3.4 ± 3.8**
Group 2	10	114 ± 80	48 ± 38**	43 ± 40**
Group 3	9	71 ± 24	161 ± 87	281 ± 165

Note. *t* test showed significant difference compared with Group 3.
* $P < 0.001$.
** $P < 0.01$.

TABLE 3
Antitumor Activities of TNF against Transplanted CAC-1 Tumor

Treatment	No. of animals	Estimated tumor volume (mean ± SD, mm³)		
		Day 13	Day 25	Day 32
Group 1	10	88 ± 64	37 ± 26*	36 ± 32*
Group 2	10	100 ± 56	33 ± 53*	35 ± 60*
Group 3	10	79 ± 34	57 ± 51*	62 ± 58*
Group 4	10	80 ± 38	348 ± 532	838 ± 821
Group 5	9	89 ± 50	689 ± 493	1882 ± 1233

Note. *t* test showed significant difference compared with Group 5.
*$P < 0.01$.

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